Ganglioside Vaccines With Emphasis on GM2

Philip Livingston

Gangliosides are neuraminic acid–containing glycosphingolipids that are anchored into the cell membrane lipid bilayer by lipophilic ceramide chains. They are overexpressed on tissues of neuroectodermal origin, and particularly in tumors such as melanomas, sarcomas, neuroblastomas, astrocytomas, and small cell lung cancers. Both active and passive immunotherapy trials have identified gangliosides as uniquely effective targets for antibody mediated melanoma immunotherapy. Induction of antibodies against GM2 by vaccination has correlated with an improved prognosis in American Joint Committee on Cancer (AJCC) stage III melanoma patients and vaccines containing GM2 chemically conjugated to keyhole limpet hemocyanin (KLH; GM2-KLH) plus the immunologic adjuvant QS-21 have proven to be consistently immunogenic. Phase III trials with this vaccine are ongoing in patients with melanoma in the United States, Canada, Europe, Australia, and New Zealand. GD2, fucosylated GM1, and GD3-KLH conjugates plus QS-21 are also consistently immunogenic, inducing IgM and IgG antibodies in the majority of patients. Polyvalent ganglioside-KLH conjugate plus QS-21 vaccines should be available in early 1999 for testing in phase II and III clinical trials.

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IN 1975, Drs Lloyd Old, Herbert Oettgen, and I initiated a series of immunization trials with melanoma and melanoma lysate vaccines mixed with various adjuvants. One hundred ten patients were immunized and the resulting serologic, delayed-type hypersensitivity (DTH), and (in the 24 patients in whom autologous melanoma cell lines were available) cytotoxic T lymphocyte (CTL) responses analyzed.[1-6] While high levels of cell-mediated cytotoxicity were detected against cultured autologous melanoma cells in some of these patients, including what was subsequently identified as human leukocyte antigen (HLA)-A2–restricted reactivity against tyrosinase in one patient, these reactivities were present before immunization.[6,7] The immunizations had no impact on cytotoxic T-cell reactivity. Vaccine-induced DTH reactions were not interpretable, since specificity could not be analyzed definitively. Serologic responses against melanoma antigens on autologous and allogeneic melanoma cells were detected in 11 patients. After extensive specificity analysis, the only antigens recognized by more than one patient were the gangliosides GM2 and GD2. Tai et al[8] also found GM2 and GD2 to be uniquely immunogenic. Ten of 26 patients vaccinated with a mix of irradiated allogeneic melanoma cell lines produced IgM antibodies against GM2 and two patients produced antibodies against GD2. Gangliosides have also been shown to be effective targets for passive immunotherapy of melanoma with monoclonal antibodies. Major clinical responses have resulted from treatment of patients with monoclonal antibodies against GM2, GD2, and GD3.[9-15] Hence, both active and passive immunotherapy trials have identified gangliosides as uniquely effective targets for melanoma immunotherapy.

Gangliosides are neuraminic acid–containing glycosphingolipids that are anchored into the lipid bilayer of the plasma membrane by their lipophilic ceramide moiety. The carbohydrate portions of gangliosides are present on the extracellular border of the plasma membrane, where they are available for recognition by antibodies. The structures of the gangliosides discussed in this review and the close proximity of the immunogenic carbohydrate epitopes to the cell membrane are demonstrated in Fig 1.

THE BASIS FOR VACCINES THAT INDUCE ANTIBODIES

Antibodies are the primary mechanism for eliminating infectious pathogens from the bloodstream. They are also ideally suited for elimination of circulating tumor cells and micrometastases. The importance of antibodies in mediating protection from tumor recurrence is well documented in experimental animals. Experiments involving administration of monoclonal antibody 3F8 against GD2 are a case in point.[16] Administration of 3F8 before intravenous tumor challenge or as late as 4 days after tumor challenge results in complete

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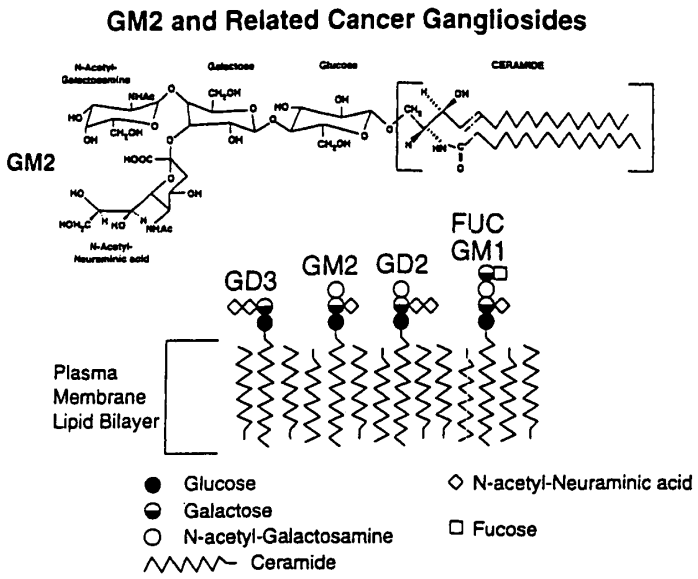

Fig 1. Demonstration of the structure of gangliosides GM2, GD2, GD3, and fucosyl GM1, and their close proximity to the external surface of the cell membrane, where they are anchored by incorporation of ceramide into the cell membrane lipid bilayer.

protection of a majority of mice. This timing may be comparable to antibody induction in or administration to patients in the adjuvant setting, after surgical resection of the primary or lymph node metastases in cancers such as melanoma and after response to chemotherapy in cancers such as small cell lung cancer (SCLC), since in both cases the targets may be circulating tumor cells and micrometastases. Administration of 3F8 seven or more days after tumor challenge had little impact on tumor progression.

There is also evidence in cancer patients that natural or passively administered antibodies in the adjuvant setting are associated with a more favorable prognosis.

(1) Natural antibodies (antibodies present in patient sera before vaccination) have been correlated with an improved prognosis. This is true for patients with paraneoplastic syndromes, in which high titers of antibodies against onconeural antigens expressed on particular cells in the nervous system and certain types of tumors have been associated both with debilitating autoimmune neurologic disorders and with delayed tumor progression and prolonged survival.[17] Also, patients with American Joint Committee on Cancer (AJCC) stage III melanoma and natural antibodies against GM2 ganglioside treated at two different medical centers have had an 80% to 100% 5-year survival rate compared with the expected rate of 40%,[18,19] as shown in Fig 2.

(2) Tumor vaccine-induced antibodies against GM2 (see Fig 2) and several other melanoma antigens at four different medical centers, and against sialyl Tn antigen in adenocarcinoma patients, have correlated with prolonged disease-free interval and survival.[19-23]

(3) Patients with Dukes C colon cancer treated with monoclonal antibody 17-1A in the adjuvant setting had a significantly prolonged disease-free and overall survival compared with randomized controls.[24]

Hence, in the adjuvant setting, passively administered and vaccine-induced antibodies have been shown to correlate with improved disease-free and overall survival in the mouse and in humans. Since the great majority of cancer patients are initially rendered free of detectable disease by surgery and or chemotherapy after initial diagnosis, vaccines that induce antibodies may have broad applicability.

EXPRESSION OF GANGLIOSIDES AT THE CELL SURFACE OF CANCERS AND NORMAL TISSUES

Ganglioside expression in a variety of malignancies has been documented by extraction, followed by thin-layer chromatography and immune thin-

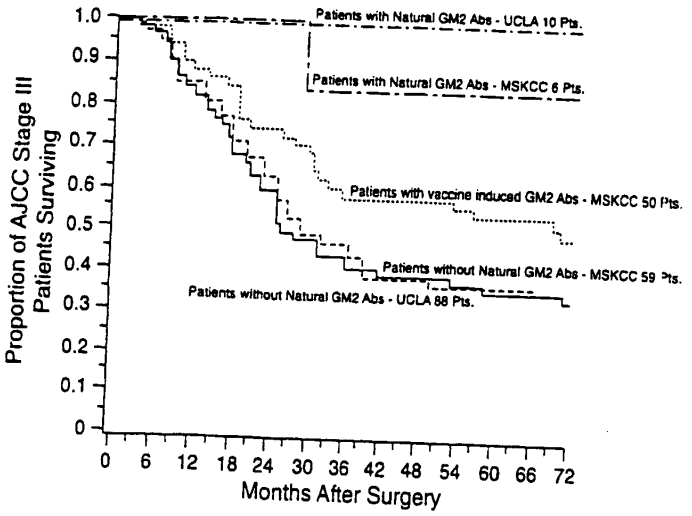

Fig 2. Correlation between the presence of natural or GM2/BCG vaccine-induced serum antibodies against GM2 and survival in AJCC stage III melanoma patients seen at the Memorial Sloan-Kettering Cancer Center (MSKCC)[11] or the UCLA School of Medicine.[18]

layer chromatography (ITLC), and by immunohistology.[25-28] These results are summarized in Table 1. GM2, GD2, and GD3, gangliosides are broadly expressed in melanomas, sarcomas, and neuroblastomas, as well as in a variety of primary brain tumors. Fucosyl GM1 expression has been largely restricted to SCLC.[27,29] 9-O-acetyl GD3 (GD3 O-acetylated at the 9 position of the terminal sialic acid) has been widely expressed only in melanomas. Surprisingly, GM2 also appears to be expressed in the majority of epithelial cancers and may represent a broadly expressed antigen.

Table 1. Expression of Gangliosides Detected by Extraction and ITLC, or by Immunohistology in >75% of Human Cancer Biopsy Specimens

Tumor	Extraction	Immunohistology	References
Melanoma	GD3, GD2, GM2, 9-O-acetyl GD3	GM2, GD3, GD2	25, 27, 63
Sarcoma	GD2, GM2, GD3	GM2, GD2, GD3	27, 59
Neuroblastoma	GD2, GM2, GD3	GM2, GD2, GD3	27, 30, 59, 60
1° brain tumors	GD2, GD3, GM2		59, 60
SCLC	FucGM1, GD2, GD3	GM2, FucGM1	27, 29, 59, 61
Epithelial cancers	GM2	GM2	27, 62

Gangliosides are also present on a variety of normal tissues.[27] GM2, GD2, and GD3 are expressed on brain cells, especially GD2, which is also expressed on some peripheral nerves. Unexpectedly, GD2 was found to be present on some B lymphocytes in the spleen and lymph nodes (but not in peripheral blood) and GM2 was detected at the secretory borders of most epithelial tissues. GD2 and GD3 were also expressed, though at lower levels, in connective tissues of multiple organs, and GD3 is known to be expressed on a subset of human T lymphocytes.[30] Fucosyl GM1 was seen in only occasional cells in the islets of Langerhans and some sensory neurons in the dorsal root ganglia.

There is now sufficient experience from clinical trials with vaccine-induced antibody responses against GM2, GD2, and several other nonganglioside antigens, and passive administration of monoclonal antibodies against GD2, GD3, and several other antigens to draw conclusions about the consequences of antigen distribution on various normal tissues. GM2, GD2, and GD3 exposure on cells in the brain and GM2 and sTn expression in cells at the secretory borders of epithelial tissues induce neither immunologic tolerance nor autoimmunity once antibodies are present. This suggests that in each case they are sequestered from the immune system. Treatment of patients with monoclonal antibodies against GD2 and GD3 has not induced CNS toxicity in children or adults. Induc-

tion of antibodies against GM2 and other antigens expressed at the secretory borders of epithelial tissues such as sTn and TF disaccharides[31-34] has not induced detectable toxicity. On the other hand, high doses of some monoclonal antibodies against GD2 have induced significant neuropathies as a consequence of GD2 expression on peripheral nerves.[15] Administration of a monoclonal antibody against Lewis X (which is expressed at the secretory borders of several epithelial tissues and also on circulating polymorphonucleosites) has resulted in no toxicity related to the epithelial tissue expression, but profound, though short-lived, neutropenia after each administration.[35,36] These two examples demonstrate that antibodies against antigens that are not sequestered from the immune system can have profound effects. Against this background, GM2, GD3, 9-O-acetyl GD3, and fucosyl GM1 all appear to be excellent targets for active immunotherapy with vaccines. Since peripheral neuropathy developed with only some monoclonal antibodies against GD2, but not others, and only at the higher doses, it may be that GD2 can also serve as a safe target for vaccine-induced antibodies.

MECHANISMS OF ANTIBODY ACTION

In general, interaction of antibody and antigen is without significance unless Fc-mediated secondary effector mechanisms are activated. On the basis of studies of bacterial infections, the most important mechanism of protection by antibodies is complement-mediated attack and lysis. IgM antibody bound to cell-surface carbohydrate antigens such as gangliosides is the most active complement activator in the intravascular space,[37,38] while IgG1 and IgG3 may be the most important extravascularly. Complement activation at the cell surface mediates inflammatory reactions, opsonification for phagocytosis, clearance of antigen-antibody complexes from the circulation, and membrane-attack complex-mediated lysis. Receptors on IgG1 and IgG3 are also the primary targets for effector cells mediating antibody-dependent cell-mediated cytotoxicity (ADCC) of tumor cells. FCδR1 (CD64), FCδR2 (CD32), and FCδR3 (CD16) receptors on a range of effector cells, including especially natural killer cells, but also T lymphocytes and cells of myeloid lineage, react with tumor cell–bound antibodies, resulting in activation of inherent cytotoxic mechanisms in the effector cells.

If antibodies of sufficient titer can be induced against cell-surface antigens to eliminate tumor cells from the blood and lymphatic systems and to eradicate micrometastases, as demonstrated in mice with antibodies against GD2,[16] this would dramatically change our approach to treating the cancer patient. With repeated showers of metastases no longer possible as a consequence of high levels of circulating antibodies, aggressive local therapies of established metastases (surgery, radiation therapy and intralesional injections) might result in long-term control of even metastatic cancers. It is also possible that complement-mediated inflammation, improved antigen presentation by specifically immune B lymphocytes, and decreased circulating tumor antigen may facilitate T-lymphocyte immunity, as has been demonstrated in other systems.[39-41]

IMMUNOGENICITY OF EARLY GM2 GANGLIOSIDE VACCINES

Initial clinical studies with whole melanoma cell vaccines demonstrating the relatively high immunogenicity of GM2 ganglioside, and the availability of purified GM2 ganglioside, were the basis for conducting a series of small clinical trials using purified GM2 for vaccine production. Serologic response against purified GM2 and tumor cells expressing GM2 was the end point.[3] Enzyme-linked immunosorbent assay (ELISA) results are summarized in Table 2. In initial trials, GM2 mixed with or adherent to the surface of various bacteria, liposomes, or proteosomes was significantly more immunogenic than GM2 alone. Of these, the proteosome and BCG vaccines were most immunogenic, inducing IgM antibodies in the majority of patients and IgG antibodies in occasional patients. Proteosome is the term used to describe preparations of the highly hydrophobic outer membrane proteins (OMP) of *Neisseria menigitidis*, which naturally form liposome-like multimolecular vesicular structures that readily incorporate antigens containing hydrophobic anchor moieties such as gangliosides.[42] In these studies, it appeared that pretreatment of patients with low-dose cyclophosphamide intravenously (300 mg/m^2), which was intended to decrease suppressor-cell activity, resulted in increased antibody titers against GM2. Overall, BCG was the most effective adjuvant. Moderate-titer IgM antibodies were induced in the majority of patients and low-titer IgG antibodies

Table 2. Peak GM2 Antibody Titer After Adjuvant Immunization of Stage III/IV Melanoma Patients With Vaccines Containing Purified $GM2^{NeuGc}$

Vaccine	Total Patients Treated	ELISA			
		IgM		IgG	
		Patients With Antibodies	Median Titer	Patients With Antibodies	Median Titer
GM2	5	0	0	0	0
GM2/R595	5	0	0	0	0
CY + GM2/R595	6	5	1/40	0	0
GM2/MPLA liposomes	6	1	1/40	0	0
GM2/proteosomes	33	22	1/160	4	1/40
GM2/BCG	5	4	1/120	0	0
CY + GM2/BCG	58	50	1/160	6	1/80
CY + GM2-KLH	6	5	1/80	0	0
CY + GM2-KLH/BCG	6	4	1/240	1	1/320
CY + GM2-KLH/Detox	6	5	1/160	0	0
CY + GM2-KLH/QS-21	9	9	1/640	8	1/160
GM2-KLH/QS-21	40	39	1/640	35	1/160

Abbreviations: ELISA, enzyme-linked immunosorbent assay; MPLA, monophosphoryl lipid A; BCG, bacillus Calmette-Guérin; KLH, keyhole limpet hemocyanin; CY, cyclophosphamide; R595, *Salmonella minnesota* mutant R595; Detox, mixture of MPLA and BCG cell-wall skeletons; QS21, purified fraction from *Quillaja saponaria* bark; proteosomes, liposome-like vesicles formed from hydrophobic *Neisseria meningitidis* outer-membrane proteins.

were induced in occasional patients. Antibody titers in most patients returned to baseline within 8 to 10 weeks after each immunization, and even with subsequent booster immunizations this pattern of antibody reactivity and duration did not change. This is consistent with GM2 acting as a T-cell–independent antigen. There was a suggestion from these initial studies that melanoma recurrence was delayed in patients developing GM2 antibody titers of ≥1/40, regardless of the adjuvant used.[34]

The expression of GM2 on most melanomas, the consistent IgM antibodies induced in patients immunized with the GM2/BCG vaccine, and the correlation of induction of GM2 antibody titers with a more favorable prognosis[34] provided the rationale for conducting a randomized trial to determine whether clinical benefit would result from vaccine-induced GM2 antibody production.[19] One hundred twenty eligible AJCC stage III melanoma patients who were free of disease after surgery were randomized to receive GM2/BCG vaccine or to receive BCG alone. All patients were pretreated with low-dose cyclophosphamide. With a minimum follow-up duration of 72 months, there was a 23% increase in the disease-free interval ($P = .004$) and a 17% increase in overall survival ($P = .03$) in patients who produced antibody titers against GM2 of 1/40 or more compared with antibody-negative patients, confirming our earlier experience (Fig 2). Comparing the treatment (GM2/BCG) and control (BCG) groups and excluding the six patients with preexisting GM2 antibodies from statistical analysis (one in the GM2/BCG group and five in the BCG group) resulted in a 17% increase in disease-free interval ($P = .02$) and a 14% increase in overall survival ($P = .15$) for patients with the GM2/BCG vaccine. However, when all patients in the two treatment groups were compared as randomized, these increases were 14% for disease-free interval and 11% for survival in the GM2/BCG treatment group, with neither result achieving statistical significance.

Although these results were encouraging, there was room for improvement. The IgM antibodies induced were of only moderate titer and short-lived. In addition, only occasional IgG antibodies against GM2 were induced. To improve the immunogenicity of the vaccine, we pursued two major lines of endeavor. Initially, we made modifications in the ganglioside structure. This was intended to permit the gangliosides to be recognized as foreign and so result in higher titer antibodies that would cross react with the original unmodified ganglioside.[46,47] After an extensive series of trials, this

approach was abandoned, because though high titer antibodies were indeed induced against the modified gangliosides, there was no cross reactivity with the unmodified ganglioside. The second approach was to augment helper T-cell reactivity and antigen processing by chemically conjugating the ganglioside to an immunogenic carrier protein and using a more potent immunologic adjuvant.

THE GM2-KEYHOLE LIMPET HEMOCYANIN CONJUGATE VACCINE PLUS QS21

Following the lead of bacterial polysaccaride vaccines that had shown that covalent attachment of antigens to immunogenic carrier proteins resulted in the highest titer antibody responses, we explored the use of ganglioside conjugate vaccines ± immunologic adjuvants.[48] Keyhole limpet hemocyanin (KLH) was the best of the six immunogenic carrier molecules tested in the mouse, the method of conjugation was crucial, and a potent immunologic adjuvant was required. GD3 conjugated via the ceramide moiety (not the carbohydrate moiety) of the ganglioside and mixed with immunologic adjuvant QS21 was optimal. QS21 is a purified homogenous saponin fraction obtained from the bark of the *Quillaja saponaria* Molina tree.[49] Simple mixture of GD3, KLH, and QS21 induced no antibodies. A variety of different carriers and adjuvants have also been tested with gangliosides GM2, GD2, and fucosyl GM1.[43,56] In each case, the ganglioside covalently attached to KLH via the ceramide moiety plus QS21 induced the highest titers of IgM and IgG antibodies. Consequently, this is the approach applied to subsequent clinical trials.

Results of initial clinical trials with the GM2-KLH conjugate vaccine plus various adjuvants are summarized in Table 2.[3] Pretreatment with cyclophosphamide had no impact on the antibody titers induced by the GM2-KLH conjugate plus QS21 vaccine. In subsequent studies, GM2 doses of 3, 10, 30 and 70 μg per vaccine were tested and the 30-μg dose selected for all future trials. In addition, it appeared that GM2-KLH epitope ratios in the conjugate of greater than 600/1 were more consistently immunogenic than lower ratios. The induced antibodies were shown to be highly specific for GM2 with minimal cross-reactivity detected against GD2 and GM3 and no cross-reactivity against other gangliosides.[43] IgG antibodies induced in immunized patients were of the IgG subclasses IgG1 and IgG3. Both IgM and IgG antibodies in most patients were able to activate complement-mediated lysis of GM2-positive, but not GM2-negative, tumor cells, and IgG antibodies from most patients were able to mediate ADCC.[50] The median duration of antibody titers of 1/40 or greater induced by the GM2-KLH plus QS21 vaccine was 6 months. Consequently, this conjugate vaccine was a clear improvement (in terms of antibodies induced) compared with the previous GM2/BCG vaccine. This provided the basis for initiating phase III clinical trials aimed at demonstrating the impact of vaccination with the GM2-KLH plus QS21 vaccine on disease-free and overall survival.

In the United States, a randomized phase III adjuvant trial comparing high-dose interferon-alfa versus the GM2-KLH plus QS21 vaccine is being conducted in patients with deep AJCC stage II primary melanomas (>4 mm depth) or stage III disease (positive regional lymph nodes) by the Eastern Cooperative Oncology Group; the Southwest Oncology Group, the North Central Cancer Treatment Group, Cancer and Leukemia Group B, Memorial Sloan-Kettering Cancer Center (MSKCC), and M.D. Anderson Cancer Center. In Europe, New Zealand, and Australia, the same patient group will be randomized to receive the same GM2 vaccine or placebo. A trial in AJCC stage II patients with thin primary tumors (2 to 4 mm) will be initiated in Europe and the United States in 1999.

IMMUNOGENECITY OF OTHER GANGLIOSIDES

GD2, GD3 and 9-O-Acetyl GD3

Using BCG, as adjuvant, we have immunized melanoma patients with GM2, GD2, GD3, GD3 lactone, and a series of O-acetyl GD3 gangliosides. The results are summarized in Table 3. GD3 was not found to be immunogenic in any patient. GD2 and GD3 lactone were found to be immunogenic in occasional patients, suggesting that with a more effective immunization approach they too might be consistently immunogenic. The O-acetyl GD3 vaccines induced antibodies that reacted with the immunizing gangliosides, but not melanoma O-acetyl GD3, which is thought to be acetylated at the 9-O position of the terminal carbon.[51] Nuclear magnetic resonance (NMR) analysis demonstrated that none of the O-acetylated GD3 preparations

Table 3. Relative Immunogenicity of Gangliosides GM2, GD2, GD3, GD3 Lactone, 9-O-Acetyl GD3, and Fucosyl GM1 in Patients Immunized With Ganglioside/BCG or Ganglioside-KLH Plus QS21 Vaccines

Vaccine	GM2		GD2		GD3		GD3 Lactone		9-O-Acetyl GD3		Fucosyl GM1	
	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
Ganglioside/BCG												
Patients vaccinated	82		12		12		9		12			
Patients making antibodies	70	16	3	0	0	0	4	0	0	0		
Median peak titer	160	80	40	0	0	0	40	0	0	0		
Ganglioside-KLH + QS21												
Patients vaccinated	30		6		7		6		6		11	
Patients making antibodies	30	24	6	0	2	0	3	5	5	5	11	10
Median peak titer	960	160	160	0	40	0	160	320	640	640	640	640

used for vaccine construction were acetylated exclusively or even primarily at the 9-O position.

We have recently immunized small groups of patients with GD3-KLH and GD3 lactone-KLH conjugate vaccines mixed with QS21. The results are summarized in Table 3. Once again, GD3 proved nonimmunogenic, but GD3 lactone succeeded in inducing antibodies against GD3, GD3 lactone, and melanoma cells expressing GD3 in the majority of patients. The basis for the increased immunogenicity of GD3 lactone over GD3 for inducing antibodies against GD3 may be the increased rigidity of GD3 lactone molecules, which is thought to increase immunogenicity, as previously described for GM3 lactone.[52] Antibodies against GD3 have also been induced in some patients by vaccination with the antiidiotype monoclonal antibody BEC2.[53] Chapman has described the induction of antibodies against GD3 in up to 30% of patients vaccinated with BEC2, depending on the adjuvant used and the route of administration.[53,54]

Trials with GD2-KLH vaccines have also succeeded in inducing antibodies detectable against synthetic GD2 by ELISA,[55] against tumor biopsy specimens by immune thin layer chromatography, and against GD2-positive cultured tumor cells by flow cytometry in the majority of patients. Analysis of these trials is still ongoing, but it is already clear that the antibodies from the majority of GD2 and GD3 lactone–vaccinated patients react with melanoma cells expressing the same antigens.

Fucosyl GM1

Eleven patients with SCLC who were free of grossly detectable disease after response to multiple cycles of chemotherapy were immunized with a fucosyl GM1-KLH plus QS21 vaccine.[56] All patients produced high-titer IgM antibodies (median titer, 1/1,280) and 10 produced moderate-titer IgG antibodies (median titer, 1/640) against fucosyl GM1. Antibodies were also reactive with SCLC biopsy-derived fucosyl GM1 by immune thin-layer chromatography, and with SCLC cell lines expressing fucosyl GM1 by flow cytometry. These results are summarized in Table 3.

FUTURE DIRECTIONS FOR GANGLIOSIDE VACCINES

While there is every indication that immunization with these single antigens may prove beneficial when administered in the adjuvant or minimal disease setting, in the long run polyvalent vaccines offer the most promise. This is because functional and antigenic heterogeneity are inherent features of malignancies and genetically based heterogeneity of responsiveness is inherent in the human immune response. Only with polyvalent vaccines can we hope to induce an immune response capable of eliminating every cancer cell. We have immunized groups of mice with mixtures of four of the individual KLH conjugate vaccines, which were either injected as a mixture in a single syringe or as individual vaccines administered to four quadrants in the same mouse, and compared immune responses to the response obtained when mice were immunized with a single one of these four components. No loss of immunogenicity was detected when four antigens were injected to the same mouse, and use of a single syringe with the four vaccines mixed together was as effective at inducing high-titer IgM and IgG antibodies against each of the peptide and carbohydrate antigens, as were the other alternatives.

We can now consistently induce IgM antibodies and in most cases IgG antibodies against GM2, GD2, and fucosyl GM1. In all cases, the antibodies react not only with the synthetic or purified immunizing ganglioside, but also with the same ganglioside obtained from tumor specimens and with cultured tumor cells. The GD3 lactone-KLH vaccine may also be ready to add to this list, but our experience with it has been quite limited. An additional GD3 lactone-KLH trial has recently been initiated to confirm our previous results. Also, we are preparing to compare the immunogenicity of GD3 lactone-KLH, with the antiidiotype monoclonal antibody BEC2 vaccine, and combinations of these two vaccines, to determine whether the combination is able to induce more consistent antibodies against GD3 and GD3-positive tumor cells than GD3 lactone-KLH or BEC2 alone.

By mid 1999, we anticipate combining the optimal single antigen vaccines described above into polyvalent vaccines. The antigens known to be expressed by different cancers as determined by extraction and immunohistology (Table 1) will guide vaccine construction. Vaccines against melanoma, neuroblastoma, and primary brain cancers will contain only the ganglioside antigens indicated, since we are not aware of any other well-defined tumor antigens that are expressed at the cell surface and are available for vaccine construction. Vaccines against neuroblastoma and SCLC on the other hand will contain the three gangliosides indicated in Table 1 in each case, but also one or more nonganglioside antigens that are known to be expressed at the cell surface of these cancers (ie, polysialic acid for neuroblastoma and polysialic acid, Globo H, and KSA for SCLC) as previously described.[27,57,58] Vaccines against epithelial cancers will contain GM2 as the only ganglioside and a variety of other carbohydrate and peptide antigens known to be expressed at the cell surface.[57,58] Once these pilot trials have demonstrated the safety and immunogenicity of these polyvalent vaccines, randomized phase III trials in the adjuvant setting will follow.

REFERENCES

1. Livingston PO, Oettgen HF, Old LJ: Specific active immunotherapy in cancer therapy, in Mihich E (ed): Immunological Aspects of Cancer Therapeutics. New York, NY, Wiley, 1982, pp 363-404

2. Livingston PO: Active specific immunotherapy in the treatment of cancer. Immunol Allergy Clin North Am 11:402-403, 1991

3. Livingston P: Approaches to augmenting the immunogenicity of melanoma gangliosides: From whole melanoma cells to ganglioside-KLH conjugate vaccines. Immunol Rev 145:147-166, 1995

4. Livingston PO: The case for melanoma vaccines that induce antibodies, in Kirkwood JM (ed): Molecular Diagnosis, Prevention and Treatment of Melanoma. New York, NY, Dekker, 1998, pp 139-157

5. Bystryn JC, Ferrone S, Livingston PO (eds): Specific immunotherapy of cancer with vaccines. Ann NY Acad Sci 690:1-330, 1993

6. Livingston PO, Shiku M, Bean MA, et al: Cell-mediated cytotoxicity for cultured autologous melanoma cells. Int J Cancer 24:34-44, 1979

7. Coulie PG, Brichard V, Van Pel A, et al: A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. J Exp Med 180:35-42, 1994

8. Tai T, Cahan LD, Tsuchida T, et al: Immunogenicity of melanoma-associated gangliosides in cancer patients. Int J Cancer 35:607-612, 1985

9. Irie RF, Matsuki T, Morton DL: Human monoclonal antibody to ganglioside GM2 for melanoma treatment. Lancet 7:786-787, 1989

10. Irie RF, Morton DL: Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside GD2. Proc Natl Acad Sci USA 83:8694-8698, 1986

11. Houghton AN, Mintzer D, Cordon-Cardo C, et al: Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: A phase I trial in patients with malignant melanoma. Proc Natl Acad Sci USA 82:1242-1246, 1985

12. Dippold WG, Bernhard H, Peter Dienes H, et al: Treatment of patients with malignant melanoma by monoclonal ganglioside antibodies. Eur J Cancer Clin Oncol 24:S65-S67, 1988

13. Raymond J, Kirkwood J, Vlock D, et al: A phase 1B trial of murine monoclonal antibody R24 (anti-GD3) in metastatic melanoma. Proc Am Soc Clin Oncol 7:A958, 1988 (abstr)

14. Cheung N-KV, Lazarus H, Miraldi FD, et al: Ganglioside GD2 specific monoclonal antibody 3F8: A phase I study in patients with neuroblastoma and malignant melanoma. J Clin Oncol 5:1430-1440, 1987

15. Saleh MN, Khazaeli MB, Wheeler RH, et al: Phase I trial of the murine monoclonal anti-GD2 antibody 14G9a in metastatic melanoma. Cancer Res 52:4342-4347, 1992

16. Zhang H, Zhang S, Cheung N-K, et al: Antibodies against GD2 ganglioside can eradicate syngeneic cancer micrometastases. Cancer Res 58:2844-2849, 1998

17. Darnell RB: Onconeural antigens and the paraneoplastic neurologic disorders: At the intersection of cancer, immunity, and the brain. Proc Natl Acad Sci USA 93:4529-4536, 1996

18. Jones PC, Sze LL, Liu PY, et al: Prolonged survival for melanoma patients with elevated IgM antibody to oncofetal antigen. J Natl Cancer Inst 66:249-254, 1981

19. Livingston PO, Wong GY, Adluri S, et al: Improved survival in stage II melanoma patients with GM2 antibodies: A randomized trial of adjuvant vaccination with GM2 ganglioside. J Clin Oncol 12:1036-1044, 1994

20. Morton DL, Foshag LJ, Hoon DS, et al: Polyvalent

melanoma vaccine improves survival of patients with metastatic melanoma. Ann NY Acad Sci 690:120-134, 1993

21. Bystryn JC, Oratz R, Roses D, et al: Relationship between immune response to melanoma vaccine immunization and clinical outcome in stage II malignant melanoma. Cancer 69:1157-1164, 1992

22. Mittelman A, Chen GZJ, Wong GU, et al: Human high molecular weight-melanoma associated antigen mimicry by mouse antiidiotypic monoclonal antibody MD2-23: Modulation of the immunogenicity in patients with malignant melanoma. Clin Cancer Res 1:705-713, 1995

23. Livingston PO, Kaelin E, Pinsky CM, et al: Serologic response in stage II melanoma patients receiving allogeneic melanoma cell vaccines. Cancer 56:2194-2200, 1985

24. Riethmuller G, Schneider-Gadicke E, Schlimok G, et al: Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. Lancet 343:1177-1183, 1994

25. Hamilton WB, Helling F, Lloyd KO, et al: Ganglioside expression on human malignant melanoma assessed by quantitative immune thin layer chromatography. Int J Cancer 53:566-573, 1993

26. Hamilton WB, Helling F, Livingston PO: Ganglioside expression on sarcoma and small-cell lung carcinoma compared to tumors of neuroectodermal origin. Proc Am Assoc Cancer Res 34:491, 1993 (abstr)

27. Zhang S, Cordon-Cardo C, Zhang HS, et al: Selection of carbohydrate tumor antigens as targets for immune attack using immunohistochemistry: I. Focus on gangliosides. Int J Cancer 73:42-49, 1997

28. Tsuchida T, Saxton RE, Morton DL, et al: Gangliosides of human melanoma. J Natl Cancer Inst 78:45-54, 1987

29. Brezicka F-T, Olling S, Nilsson O, et al: Immunohistological detection of fucosyl- G_{m1} ganglioside in human lung cancer and normal tissues with monoclonal antibodies. Cancer Res 49:130-1305, 1989

30. Meritt WD, Taylor BJ, Der-Minassian V, et al: Coexpression of GD_3 ganglioside with CD45RO in resting and activated human T lymphocytes. Cell Immunol 173:131-148, 1996

31. MacLean GD, Reddish MA, Koganty RR, et al: Antibodies against mucin-associated sialyl-Tn epitopes correlate with survival of metastatic adenocarcinoma patients undergoing active specific immunotherapy with synthetic STn vaccine. J Immunother 19:59-68, 1996

32. Springer GF: T and Tn, general carcinoma autoantigens. Science 224:1198-1206, 1984

33. Adluri S, Helling F, Calves MJ, et al: Immunogenicity of synthetic TF- and sTn-KLH conjugates in colorectal carcinoma patients. Cancer Immunol Immunother 41:185-192, 1995

34. Livingston PO, Ritter G, Srivastava P, et al: Characterization of IgG and IgM antibodies induced in melanoma patients by immunization with purified GM2 ganglioside. Cancer Res 49:7045-7050, 1989

35. Mordoh J, Silva C, Albarellos M, et al: Phase I clinical trial in cancer patients of a new monoclonal antibody FC-2.15 reacting with proliferating tumor cells. J Immunother 17:151-180, 1995

36. Capurro M, Bover L, Portela P, et al: FC-2.15, a monoclonal antibody active against human breast cancer, specifically recognizes Lewis X hapten. Cancer Immunol Immunother 45:334-339, 1998

37. Raff HV, Bradley C, Brady W, et al: Comparison of functional activities between IgG1 and IgM class-switched human monoclonal antibodies reactive with group B streptococci or *Escherichia coli* K1. J Infect Dis 163:346-352, 1991

38. Wolff EA, Esselstyn J, Maloney G, et al: Human monoclonal antibody homodimers. Effect of valency on in vitro and in vivo antibacterial activity. J Immunol 148:2469-2474, 1992

39. Lin R-H, Mamula MJ, Hardin JA, et al: Induction of autoreactive B cells allows priming of autoreactive T cells. J Exp Med 173:1433-1439, 1991

40. Serreze DV, Chapman HD, Varnum DS, et al: B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: Analysis of a new "speed congenic" stock of NOD.Igμnull mice. J Exp Med 184:2049-2053, 1996

41. Sopori ML, Donaldson LA, Savage SM: T lymphocyte heterogeneity in the rat. III. Autoreactive T cells are activated by B cells. Cell Immun 128:427-437, 1990

42. Livingston PO Calves MJ, Helling F, et al: GD3/proteosome vaccines induce consistent IgM antibodies against the ganglioside GD3. Vaccine 11:1199-1204, 1993

43. Helling F, Zhang A, Shang A, et al: GM2-KLH conjugate vaccine: Increased immunogenicity in melanoma patients after administration with immunological adjuvant QS-21. Cancer Res 55:2783-2788, 1995

44. Livingston PO, Natoli EJ Jr, Jones Calves M, et al: Vaccines containing purified GM2 ganglioside elicit GM2 antibodies in melanoma patients. Pro Natl Acad Sci USA 84:2911-2915, 1987

45. Livingston PO, Adluri S, Helling F, et al: Phase I trial of immunological adjuvant QS-21 with a GM2 ganglioside-KLH conjugate vaccine in patients with malignant melanoma. Vaccine 12:1275-1280, 1994

46. Ritter G, Boosfeld E, Adluri R, et al: Antibody response to immunization with ganglioside GD3 and GD3 congeners (lactones, amide and ganglioisidol) in patients with malignant melanoma. Int J Cancer 48:379-385, 1991

47. Ritter G, Ritter-Boosfeld E, Adluri R, et al: Analysis of the antibody response to immunization with purified O-acetyl GD3 gangliosides in patients with malignant melanoma. Int J Cancer 62:1-5, 1995

48. Helling F, Shang Y, Calves M, et al: Increased immunogenicity of GD3 conjugate vaccines: Comparison of various carrier proteins and selection of GD3-KLH for further testing. Cancer Res 54:197-203, 1994

49. Kensil CR, Patel U, Lennick M, et al: Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. J Immunol 146:431-437, 1991

50. Livingston PO, Zhang S, Walberg L, et al: Tumor cell reactivity mediated by IgM antibodies in sera from melanoma patients vaccinated with GM2-KLH is increased by IgG antibodies. Cancer Immunol Immunother 43:324-330, 1996

51. Thurin J, Herlyn M, Hindsgaul O, et al: Proton NMR and fast atom bombardment mass spectrometry analysis of the melanoma associated ganglioside 9-0 acetyl-GD3. J Biol Chem 260:14556-14563, 1985

52. Nores GA, Dohi T, Taniguchi M, Hakomori S-I: Density dependent recognition of cell surface GM3 by a certain anti-melanoma antibody, and GM3 lactone as a possible immunogen. J Immunol 139:3171-3176, 1987

53. Chapman PB, Livingston PO, Morrison ME, et al:

Immunization of melanoma patients with antiidiotypic monoclonal antibody BEC2 (which mimics GD3 ganglioside): Pilot trials using no immunological adjuvant. Vaccine Res 3:59-69, 1994

54. Chapman PB: Anti-idiotypic monoclonal antibody cancer vaccines. Semin Cancer Biol 6:367-374, 1995

55. Chapman PB, Meyers M, Williams L, et al: Immunization of melanoma patients with a bivalent GM2/GD2 ganglioside conjugate vaccine. Proc Am Assoc Cancer Res 39:369, 1998 (abstr)

56. Dickler MN, Grant SC, Ragupathi G, et al: Vaccination with fucosyl-GM1 (fucGM1) keyhole limpet hemocyanin (KLH) conjugate plus QS-21 in patients with small cell lung cancer (SCLC) after a major response to therapy. Proc Am Soc Clin Oncol 17:1671, 1998 (abstr)

57. Zhang S, Zhang H-H, Cordon-Cardo C, et al: Selection of tumor antigens as targets for immune attack using immunohistochemistry: III. Protein antigens. Clin Cancer Res (in press)

58. Zhang S, Cordon-Cardo C, Zhang HS, et al: Selection of carbohydrate tumor antigens as targets for immune attack using immunohistochemistry: Blood group-related antigens. Int J Cancer 73:50-56, 1997

59. Hamilton WB, Helling F, Livingston PO: Ganglioside expression on sarcoma and small cell lung cancer compared to tumors of neuroectodermal origin. Proc Am Assoc Cancer Res 34:491, 1993 (abstr)

60. Helling F, Livingston PO: Ganglioside conjugate vaccines: Immunotherapy against tumors of neuroectodermal origin. Mol Chem Neuropathol 21:299-309, 1994

61. Nilsson O, Mansson J-E, Bretcicka T, et al: Fucosyl-G_{m1}—A ganglioside associated with small cell lung cancer carcinomas. Glycoconjugate J 1:43-49, 1984

62. Nishinaka Y, Ravindranath MH, Irie RF: Development of human monoclonal antibody to ganglioside G_{M2} with potential for cancer treatment. Cancer Res 56:5666-5671, 1996

63. Tsuchida T, Saxton E, Morton D-L, et al: Gangliosides of human melanoma. J Natl Cancer Inst 78:45-54, 1987